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(54) Title: MAC-1 I-DOMAIN PROTEIN USEFUL IN BLOCKING ADHESION AND MIGRATION OF NEUTROPHILS

(57) Abstract

An isolated and purified protein of the I-Domain from the human leukocyte β_2-integrin Mac-1, expressed in recombinant *Escherichia coli* as a soluble fusion protein with glutathione S-transferase (GST). The protein, a functional derivative, fragment, analog or chemical derivative of such fragment is useful in the treatment of inflammation by interfering with the cell adhesion mechanism to block adhesion and migration of neutrophils.

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Mac-1 I-Domain Protein Useful in Blocking Adhesion and Migration of Neutrophils

BACKGROUND OF THE INVENTION

Cell adhesion molecules are essential in a number of cellular processes including immunity and inflammation, cell anchorage and migration, and cell growth and differentiation. Among the large number of various cell adhesion proteins, the leukocyte integrins are involved in mediating the adhesion and endothelial trans-migration of leukocytes into inflamed tissue. These integrins are membrane-anchored proteins on the surface of the leukocytes, serving as receptors to various ligands. These receptors are heterodimer proteins consisting of alpha and beta subunits, and since the 3 known leukocyte integrins share a common beta-2 subunit, they are also called beta-2 integrins. In these 3 β2 integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18), the β subunit CD18 interacts with the α subunit CD11 in a noncovalent manner.

Both the α and β subunits of these integrins are large glycoproteins; the CD18 subunit is about 95 kd in molecular mass. The 170 kd α subunit CD11b in Mac-1 contains an I-domain (or A-domain) which is also conserved in CD11a, CD11c, and a few other integrins (Larson *et al*, J. Cell. Biol., 108:703-12 (1989)). This I-domain of about 200 amino acid residues appears to be an insertion sequence; it is not found in some other integrins. The I-domain exhibits sequence similarity to the domains involved in ligand binding of proteins such as von Willebrand factor, cartilage matrix protein, and complement proteins C2 and factor B.

Thus it is intriguing to consider that the I-domain in the β2 integrins may play a role in the interaction of these integrins to their ligands. Results suggesting the I-domain might function as a recognition region in ligand binding were reported by Diamond *et al*, J. Cell Biol., 120:1031-43 (1993); they showed that monoclonal antibodies to CD11b I-domain could block the binding of Mac-1 to iC3b, fibrinogen, ICAM-1 and a neutrophil ligand. It is known that Mac-1 ligand binding requires the presence of cation. Michishita *et al*, Cell, 72:857-67 (1993) demonstrated the binding of Mn^{+2} to a *E. coli*-derived recombinant I-domain of CD11b, again suggesting the involvement of the I-domain in ligand binding.

To provide CD11b I-domain to study its function and structure, *E. coli* expression of the recombinant protein was required. Unfortunately, expression levels of the I-domain by itself were low, and the protein was difficult to purify. Attempts were made to generate a strain which expressed, at high levels, the I-domain as a fusion to GST (glutathione S-transferase), with a thrombin cleavage site

at the fusion junction so that the I-domain could be recovered from the fusion product. However this fusion protein was not susceptible to thrombin proteolysis.

The present invention demonstrates that producing recombinant proteins in *E. coli* as fusions to GST has some of the following advantages: 1) GST itself is expressed at high levels and therefore should promote high level production of the fusion protein, 2) GST itself is highly soluble and can serve to facilitate/stabilize the folding of the fusion protein, leading to a soluble and active product, and 3) GST can be used as a purification handle, i.e., one-step purification of the fusion protein by affinity chromatography on immobilized glutathione, using a glutathione Sepharose 4B column.

The present construct overcomes problems caused by the physical close proximity of the GST and I-domain, masking the accessibility of the cleavage site. The present construct introduces residues such as glycine at the GST and I-domain fusion point to separate the two structures. In addition, the present invention teaches a GST and I-domain fusion with a Factor X^a cleavage site to compare the efficiency of thrombin and Factor X^a proteolysis in such fusion products. The different fusion junctions linking GST and I-domain are shown below. The residues underlined are the recognition sequence for thrombin or Factor X^a with the arrow(-↑-) indicating the cleavage point. The residues highlighted in boldface are the extensions at the N-terminus of I-domain after proteolysis. Because of the requirements for recognition by the protease, extra residues in addition to the desired 2 glycines are necessary for extension of the I-domain from the fusion constructs.

Thrombin

GST--Leu-Val-Pro-Arg-↑-Gly-Ser--(Ser133...Glu337)

Thrombin

GST--Leu-Val-Pro-Arg-↑-Gly-Ser-**Gly-Gly**--(Ser133...Glu337)

Factor X^a

GST--Ile-Glu-Gly-Arg-↑-Gly-Ile-Pro-Gly-Gly--(Ser133...Glu337)

Note that in the subject recombinant product, the CD11b I-domain sequence is that defined by the serine at position #133 ending at the glutamic acid at #337. In the report of Michishita *et al* (cited, above), their *E. coli*-derived recombinant CD11b I-domain (called the A domain) starts at glycine at #111 and ends with alanine at #318 (see Figure 1). Without engineering for the exact I-domain

sequence, Michishita *et al* took advantage of the available restriction enzyme sites to clone a sequence coding for the region around the I-domain, thus shifting the I-domain towards the N-terminus of CD11b.

Uniquely, the addition of two glycine residues between the GST and I-domain in the subject construct does allow thrombin processing of the fusion protein. The construct with the Factor X^a cleavage site also can be processed by Factor X^a efficiently, giving a better yield of I-domain than that from the fusion with thrombin cleavage. The subject invention provides cell extracts prepared from the *E. coli* strain producing the fusion protein with the X^a cleavage site for downstream isolation of the CD11b I-domain.

INFORMATION DISCLOSURE STATEMENT

European Patent Application 0365837 (Springer et al.) discloses the general background for intercellular adhesion molecules (ICAM-1) and their function derivatives which may be useful in the treatment of inflammation.

European Patent Application 0391088 (Springer et al.) discloses the intercellular adhesion molecules (ICAM-1) and their function derivatives which may be useful in the treatment of viral infections.

European Patent Application 0364690 (Springer et al.) discloses the leukocyte adhesion receptor Mac-1 alpha subunit and corresponding DNA and derivatives which may be useful in the treatment of inflammation.

European Patent Application 0387668 (Springer et al.) discloses the intercellular adhesion molecules classified as ICAM-2 which are described to be involved in the process where lymphocytes migrate to inflammation sites.

Diamond et al., "The I-domain is a Major Recognition Site on the Leukocyte Integrin Mac-1 (CD11b/CD18) for Four Distinct Adhesion Ligands, J. of Cell Biology, **120**, 4, 1031-1043 (1993) discloses that mAbs specific for the I-domain block Mac-1-dependent adhesion.

Michishita et al., "A Novel Divalent Cation-Binding Site in the A Domain of the Beta-2 Integrin CR3 (CD11/CD18) Is Essential for Ligand Binding", Cell, **72**, 857-867 (March 26, 1993) discloses that certain mutations in the I-domain region of Mac-1 block adhesion to iC3b.

Zhou et al., "Differential Ligand Binding Specificities of Recombinant CD11b/CD18 Integrin I-Domain", J. Biological Chemistry, **269**, 25, 17075-17079 (June 24, 1994) report the expression of a recombinant form of the I-domain of CD11b and that this domain binds fibrinogen and ICAM-1 but that it does not

recapitulate the entire CD11b/cd18 ligand repertoire.

Kern et al., "The Role of the I Domain in Ligand Binding of the Human Integrin $\alpha_1\beta_1$", J. Biological Chemistry, **269**, 36, 22811-22816 (September 9, 1994) report that the I Domain plays a central role in ligand recognition for all integrin α subunits containing this domain.

Muchowski et al., "Functional Interaction between the Integrin Antagonist Neutrophil Inhibitory Factor and the I Domain of CD11b/CD18", J. Biological Chemistry, **269**, 42, 26419-26423 (October 21, 1994) report that recombinant neutrophil inhibitory factor (rNIF) associates with the about 200 amino acid residue I domain of CD11b/CD18 and that this intereaction is essential for inhibition of neutrophil function by NIF.

U.S. Patent 5,091,303 to Arnaout et al. discloses a 29kD neutrophilic protein which binds to autoantibodies present in the sera of individuals afflicted with Wegener's granulomatosis and methods using these autoantibodies to diagnose individuals afflicted with Wegener's granulomatosis.

U.S. Patent 5,200,319 to Arnaout et al. discloses a 29kD neutrophilic protein which is used in a method of diagnosing pauci-immune nectrotizing and/or crescentic glomerulnephritis in a patient.

SUMMARY OF THE INVENTION

In one aspect the subject invention is a fusion protein of glutathione-S-transferase (GST) and I-Domain derived from human leukocyte B_2-integrin Mac-1 in which the GST and the I-Domain are linked by a peptide segment containing a Factor X^a cleavage site as set forth in ID SEQ NO: I. This fusion protein provides an improved means for handling the I-Domain protein for synthesis and expression and a unique cleavage site which provides accessability for cleavage by a Factor X^a enzyme.

In another aspect, the invention is an I-Domain protein defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I. This sequence contains the I-Domain and the above mentioned special cleavage site.

In yet another aspect, the invention is a pharmaceutical composition comprising the recombinant I-Domain protein derived from human leukocyte B_2-integrin Mac-1 defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I; and a pharmaceutically acceptable carrier or excipient. The pharmaceutical composition can also consist of a fragment, analog or chemical derivative of the aforementioned

I-Domain protein.

In yet another aspect, the invention is a method for treating inflammation comprising

the administration to a patient suffering from an inflammatory condition a pharmaceutically effective amount of an anti-inflammatory agent comprising a recombinant I-Domain protein derived from human leukocyte B_2-integrin Mac-1 defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I. The method can also consist of a fragment, analog or chemical derivative of the aforementioned recombinant I-Domain protein.

The fusion protein, I-domain protein (which can contain the special cleavage segment) and the pharmaceutical compositions prepared therefrom are all recombinant proteins substantially free of contaminants or other biological impurities.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1. Schematic representation of the A-domain described by Michishita et al., "A Novel Divalent Cation-Binding Site in the A Domain of the Beta-2 Integrin CR3 (CD11/CD18) Is Essential for Ligand Binding", Cell, **72**, 857-867 (March 26, 1993) and the I-domain of the present invention. Note that the A-domain is about the same size as the I-domain, but begins about 20 amino acids upstream in the sequence of Mac-1 (Gly_{111}). Accordingly, it ends at Ala_{318}, some 20 residues upstream of the C-terminal residue in the I-domain (Glu_{337}). The A-domain has a C-terminal extension of Asn-Ser-Ser, introduced as part of the cloning strategy. The I-domain is N-terminally extended by H_2N-Gly-Ile-Pro-Gly-Gly-.. , a sequence required to promote cleavage by Factor X^a . The first Mac-1 residue in the I-domain construct is the serine corresponding to Ser_{133}.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is an isolated and purified protein corresponding to the I-domain from the human leukocyte β_2-integrin Mac-1, expressed in recombinant *Escherichia coli* as a soluble fusion protein with glutathione S-transferase (GST). The protein is useful in the treatment of inflammation by interferring with the cell adhesion mechanism to block adhesion and migration of neutrophils.

The fusion protein corresponding to the glutathione-S-tranferase (GST) and the I-Domain from the human leukocyte β_2-integrin Mac-1, is shown as a complete amino acid sequence (SEQ ID NO: I). The I-Domain protein amino acids are

numbered according to their location in the fusion protein. The NH_2-terminal portion of the molecule (residue 220) is glutathione-S-transferase (GST), a fusion partner which helps increase the level of soluble expression, and facilitates purification by GSH-affinity chromatography. Residues from 221-230 indicate the segment linking GST to the I-domain and the site of cleavage by Factor X^a at Arg225 - Gly226 is dictated by the sequence; [Ile-Glu-Gly-Arg-↓-Gly-Ile-Pro-]. The boldface Gly-Gly sequence represents a spacer to allow accommodation of the proteinase to the site of cleavage at Arg-Gly. The Ser at 231 following the Gly-Gly sequence corresponds to the beginning of the I-domain, Ser_{133} in processed Mac-1, and the construct ends with Glu_{337}. Accordingly, the I-domain begins with the pentapeptide sequence: Gly-Ile-Pro-Gly-Gly- required to allow removal of the GST by Factor X^a, followed by the I-domain of Mac-1 (residues 133 through 337). This structure is schematicized in Fig. 1.

The isolated protein is useful for the treatment of inflammation and related conditions in human patients and other warm blooded animals by either parenteral or oral administration.

Pharmaceutical compositions of this invention may be prepared by combining the protein of the I-domain from the human leukocyte β_2-integrin Mac-1 (SEQ ID NO: I), fragment, variant, analog or chemical derivative thereof of this invention with pharmaceutically acceptable carrier, pharmaceutically acceptable adjuvants or excipients employing standard and conventional techniques.

Preferably, the pharmaceutical composition is prepared using conventional techniques in unit dosage form containing an anti-inflammatory effective or appropriate amounts of the active ingredient, protein, that is, I-domain from the human leukocyte β_2-integrin Mac-1 (SEQ ID NO: I), fragment, variant, analog or chemical derivative thereof.

The quantity of active component, according to this invention, in the pharmaceutical composition and unit dosage form thereof may be varied or adjusted widely depending upon the particular application, the potency of the particular compound or the desired concentration. In therapeutic use for treating, or combatting inflammation or any of its related symptoms in patients that can be diagnosed with such, the isolated and purified recombinant protein or pharmaceutical compositions thereof will be administered orally and/or parenterally at a dosage to obtain and maintain a concentration, that is, an amount, or blood-level of active component in the patient undergoing treatment which will be anti-inflammatorially effective. Generally, such pharmaceutically effective amount of

dosage of active component will be evidenced by monitoring of the inflammatory site. It is to be understood that the dosages may vary depending upon the requirements of the patient, the severity of the inflammation being treated, and the particular component being used. Also, it is to be understood that the initial dosage administered may be increased beyond a predetermined upper level in order to rapidly achieve the desired blood-level or the initial dosage may be smaller than the optimum and the daily dosage may be progressively increased during the course of treatment depending on the particular situation. If desired, the daily dose may also be divided into multiple doses for administration, e.g., two to four times per day.

The compositions of this invention can be administered parenterally, i.e., by injection, for example, by intravenous injection or by other parenteral routes of administration. Pharmaceutical compositions for parenteral administration will generally contain a pharmaceutically acceptable amount of the recombinant I-domain protein from the human leukocyte β_2-integrin Mac-1 (SEQ ID NO: I), fragment, variant, analog or chemical derivative thereof mixed in a pharmaceutically acceptable liquid carrier such as, for example, water-for-injection and a buffer to provide a suitably buffered isotonic solution.

The active component will be admixed in the carrier in an amount sufficient to provide a pharmaceutically acceptable injectable concentration. The resulting liquid pharmaceutical composition will be administered so as to obtain the above-mentioned anti-inflammatory effective amount of dosage.

The fusion protein construct of the subject invention utilizes the GST partner to facilitate high levels of expression of soluble protein, and to allow purification by affinity chromatography on immobilized glutathione (GSH). It was also designed to contain an accessible Factor X^a-sensitive site in the region linking GST to the I-domain so as to permit removal of the N-terminally attached GST moiety. Affinity chromatography of *E. coli* extracts over a column of GSH-Sepharose allowed separation of the bound GST/I-domain from contaminating proteins which passed directly through the column. Removal of the GST/I-domain was effected by washing the column with buffer containing GSH. The fusion protein thus purified was hydrolyzed with Factor X^a which cleaved specifically in the linker region, and the resultant GST and I-domain proteins were separated by ion-exchange chromatography on a column of S-Sepharose. The final I-domain product was shown to be >99% pure by sequence and compositional analysis, and by electrospray ionization mass spectroscopy which gave a molecular weight in agreement with that expected for the I-domain (25,767). The fact that the I-domain was resistant to

trypsin, coupled with physical characterization by circular dichroism and two-dimensional NMR provided evidence that the recombinant I-domain has a folded and ordered three dimensional structure.

Because of the enormous size and complexity of the β_2-integrins, they are not amenable to structural analysis by conventional methods. The I-domain, however, represents a reasonable target; it is relatively small in size, has no disulfide cross links, only low-level of glycosylation and, most importantly, it appears to be functionally relevant. In preparation for preparing the subject I-domain product, the I-domain of Mac-1 in *E. coli* was cloned and expressed. Two I-domain constructs were designed. One had an extension at the COOH-terminus of six histidines (6His tag) to facilitate purification by immobilized metal ion affinity chromatography. This construct begins with H_2N-Ser_{133}-Asp-Ile-Ala-Phe-Leu..., and ends with ..Ser-Gln-Glu_{337}-Ile-Leu-Gly-Arg-↓-His-His-His-His-His-His-COOH, where the numbers indicate residues in the Mac-1 α-subunit (SEQ ID NO: I, Fig. 1), and the arrow indicates a bond designed, by virture of the preceeding 4 amino acids, to be a cleavage point for Factor X^a. Throughout this description, the numbering system will be that of the processed Mac-1, lacking the signal peptide, a nomenclature consistent with that of Michishita *et al.*, Cell, 72:857-67 (1993). These findings helped promote interest in the recombinant I-domain.

Another construct was made which contained GST as a fusion protein. The general layout of this molecule is as shown below:

GST-Leu-Val-Pro-Arg-↓-Gly-Ser-Ser_{133}.....Glu_{337}-Ile-Glu-Gly-Arg-↑-$(His)_6$

where ↓ indicates a thrombin cleavage sequence, and ↑ indicates a Factor X^a cleavage sequence. Accordingly, the I-domain is defined, as for the 6His construct, as the sequence from Ser_{133} and Glu_{337}. This construct, therefore, had two handles for purification, GST and the 6His tag. Unfortunately, this protein could not be cleaved with thrombin, Factor X^a, or even trypsin. It appeared that the thrombin site was limited in accessibility, due to hindrance in the inter-domain region.

To solve this problem, a new set of constructs was designed in which the 6His tag was removed, and where additional amino acids were inserted in the linker region connecting GST and the I-domain, thus:

Thrombin construct: GST-Leu-Val-Pro-Arg-↓-**Gly-Ser-Gly-Gly**-Ser_{133}....Glu_{337}-COOH

Factor X^a construct: GST-Ile-Glu-Gly-Arg-↑-**Gly-Ile-Pro-Gly-Gly**-Ser_{133}...Glu_{337}-COOH

Again, arrows indicate sites of processing by the two proteases and therefore

the constructs had N-terminal extensions highlighted in boldface, but had no COOH-terminal extensions. Both fusion proteins were found to be processed by thrombin and Factor X^a, respectively, but the latter proved to be much better in its yield of I-domain.

Cloning and Expression of Two Fusion Proteins of CD11b I-domain to GST

Two constructs were made for the expression of the CD11b I-domain as a fusion to GST in the cytoplasm of *E. coli*. One construct has the recognition site for Factor X^a cleavage at the fusion junction, while the other construct has the thrombin proteolysis site. After purification of the fusion proteins, the I-domain can be recovered from the fusion protein by Factor X^a or thrombin processing. In order to separate the I-domain from the GST structure to allow accessibility of the proteolytic site, two glycine residues were introduced into the fusion segment. Due to the proteolytic recognition sequence it was necessary to introduce other residues in addition to the two glycine residues. After proteolytic processing, the CD11b I-domain has the N-terminus extension shown in boldface. The CD11b I-domain contains amino acid residues from Ser133 to Glu337.

Factor X^a cleavage product: **Gly-Ile-Pro-Gly-Gly**-(Ser133....Glu337)

Thrombin cleavage product: **Gly-Ser-Gly-Gly**-(Ser133....Glu337)

Two DNA sequences coding for the CD11b I-domain were cloned by PCR from plasmid pET-CD11b/I$(His)_6$, as described in the EXAMPLE. These BamHI and EcoRI digested 660 bp DNA fragments were cloned into the Pharmacia expression vectors pGEX-3X and pGEX-2T which were digested with BamHI and EcoRI. The 660 bp fragment from PCR primers KAC250 and KAC251 was cloned into pGEX-3X and the one from primers KAC249 and KAC251 was cloned into pGEX-2T. The resulting expression plasmid vectors carrying the I-domain fused to GST are named pGST-X^a-CD11b/I and pGST-Throm-CD11b/I, with the Factor X^a cleavage site and thrombin process site, respectively. In the expression plasmids, the fusion protein is under the control of the strong tac promoter which can be induced by IPTG. The plasmids also carry the *lacI*^{q} sequence to repress the tac promoter activity in the absence of an inducer such as IPTG. The presence of the repressor *lacI*^{q} is important for minimizing background expression of the recombinant protein, especially in cases where the recombinant protein is detrimental to the host cell. If the *lacI*^{q} repressor sequence is not present in the plasmid, then one would want to maintain the plasmid in a *E. coli* host carrying the repressor, usually on the F' episome. Since the above vectors carry *lacI*^{q}, the *E. coli* DH1 used for plasmid construction and *E. coli* K12S used for expression do not contain the repressor sequence.

To test the expression of the fusion protein from the above expression vectors, pGST-X^a-CD11b/I and pGST-Throm-CD11b/I were transformed into the 3 *E. coli* hosts JM103, JM109 and K12S. These strains were induced for expression of the fusion protein by $1x10^{-3}$M IPTG as described in the EXAMPLE. When cell extracts were analyzed by SDS-PAGE, a prominent protein band corresponding to the expected size of the fusion protein (about 45 kd) was observed. This protein band is absent in cell extracts derived from strains expressing GST without the I-domain fusion. As expected, GST is expressed at high levels in these control extracts. The expression of the fusion protein in these 3 hosts with induction by $1x10^{-3}$M IPTG was repeated more than once. It appears that JM109 and K12S are better hosts than JM103, producing high levels of the fusion protein, leading to the accumulation of fusion protein as 30% of total cell protein.

By inducing expression with $1x10^{-3}$M IPTG, the majority of the fusion product accumulates in an insoluble, aggregated form, i.e., inclusion bodies. In some cases a recombinant protein can remain in soluble form if the promoter is turned on slowly, allowing protein synthesis which might lead to a more favorable condition for the folding of the recombinant protein into soluble state. The distribution of the GST I-domain fusion protein in soluble and insoluble forms under promoter induction at various levels of IPTG was examined. As the IPTG level is lowered from $1x10^{-3}$ M, $1x10^{-4}$ M, $5x10^{-5}$ M to $1x10^{-5}$ M, more of the fusion product accumulates in the soluble state. At $1x10^{-5}$ M IPTG, about 60-80% of the fusion protein produced are in the soluble state.

Using the *E. coli* strain K12S(pGST-X^a-CD11b/I) which produces the I-domain fusion to GST with the Factor X^a cleavage site, more than 25 liters of cells can be grown in shake-flasks with $1x10^{-5}$M IPTG to induce expression. From these cells, cell extracts are prepared by sonication and supernatant extracts free of inclusion bodies can be obtained by centrifugation to remove the insoluble material. These supernatant extracts can be purified for the I-domain. Over 10 mg of purified I-domain can be obtained from 1 liter of cells. This amount of production is significant, since shake-flasks are used to grow *E. coli* cultures, which reach a cell density of only about 1.5 A550.

In order to label the fusion protein with ^{15}N and $^{15}N/^{13}C$ for isolation of labeled I-domain for NMR analysis the cell growth conditions were tested to determine what would efficiantly but economically produce the labeled protein. Pilot experiments involved using the Celtone-N medium (without ^{15}N or ^{13}C, see EXAMPLE). The unlabeled Celtone-N medium was diluted with unlabeled M9 salts

medium at a ratio of 1:1, 1:2 and 1:3 and used to culture strain K12S(pGST-X^a-CD11b/I) with $1x10^{-5}$M IPTG for induction of expression. Celtone medium itself appears to give lower expression levels (24% of total cell protein) than the Luria Broth (30% of total cell protein). Dilution of the Celtone medium with 1 part or 2 parts of M9 salts medium does not significantly decrease expression, while dilution with 3 parts of M9 did lower expression. Under these culture conditions, about 60% of the fusion product remains in the soluble state.

Using the ^{15}N- and $^{15}N/^{13}C$-containing Celtone medium diluted with 2 parts of ^{15}N-and $^{15}N/^{13}C$-containing M9 salts medium (see, EXAMPLE), several liters of the K12S(pGST-X^a-CD11b/I) cells were grown (the strain expressing I-domain fusion to GST with Factor X^a cleavage site) and prepared supernatant extracts free of inclusion bodies. The ^{15}N- and $^{15}N/^{13}C$-labeled I-domain molecules were isolated for NMR analysis.

The recombinant I-domain of the α-subunit CD11b of human leukocyte integrin Mac-1 was produced in *E. coli* as a fusion to GST (glutathione S-transferase), under the control of the tac promoter in a pBR-based vector background. High level expression leads to the accumulation of the fusion product to at least 30% of total cell protein. Induction of the tac promoter with high levels of IPTG results in the majority of the fusion product in insoluble, aggregated inclusion body form. By lowering the IPTG level to $1x10^{-5}$M, over 60% of the fusion protein produced remains in a soluble form in the cytoplasm of *E. coli*.

Two fusion proteins were produced, each with a specific proteolytic cleavage site (Factor X^a or thrombin) at the fusion junction to allow the recovery of the I-domain from the fusion product by digestion with either Factor X^a or thrombin. To assure that the I-domain and GST are not in too close physical proximity to mask the accessibility of the cleavage site to the protease, glycine residues were introduced into the fusion junction to separate the two polypeptide structures. After cleavage, the resulting I-domain has 4-5 extra residues at the N-terminus. To provide starting material for purification of the I-domain, multi-liters of *E. coli* cells were cultured in shake-flasks, and inclusion body-free cell extracts prepared.

EXAMPLE

Enzymes and Basic Molecular Biology Techniques

Restriction endonucleases, other DNA modifying enzymes and T4 DNA ligase were from New England Biolabs or Boehringer Mannheim. All enzymes were used according to the manufacturer's instructions.

All plasmids used carry the ampicillin-resistance marker and were constructed and maintained in *E. coli* DH1 in the presence of 100 µg/ml ampicillin in either Luria broth or on Antibiotic Medium #2 (Difco) agar plates. Isolation of DNA fragments, transformation, small and large scale plasmid preparation, and other basic molecular biology techniques were according to those described by Sambrook *et al* , Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (1989). Verification of the plasmid constructs was by restriction enzyme analysis and DNA sequencing using the Sequenase kit from United States Biochemical Corporation.

Construction of Vectors for Expression of CD11b I-domain Fused to GST

The vectors, pGEX-3X and pGEX-2T, used for constructing plasmids with the CD11b I-domain sequence fused to GST, were purchased from Pharmacia. Plasmid pGEX-3X carries the Factor X^a cleavage recognition site and pGEX-2T the thrombin cleavage site in the polylinker region downstream from the GST sequence. These vectors contain the tac promoter [inducible by IPTG (isopropylthiogalactoside)] to control GST expression, the *lacI*^{q} repressor sequence to minimize the tac promoter activity in the absense of IPTG, a polylinker region for gene cloning as a fusion to GST, the ampicillin-resistance marker, and the replication origin derived from pBR322.

The CD11b I-domain sequence was cloned from a plasmid containing human CD11b cDNA which was originally constructed by Corbi *et al*, J. Biol. Chem., 263:12403-11 (1988). With the pET-CD11b/I$(His)_6$ as the PCR template, oligonucleotides named KAC250 and KAC251 were used to clone the I-domain sequence for fusion with GST in pGEX-3X, while KAC249 and KAC251 were used to clone the I-domain for fusion with GST in pGEX-2T. The sequences of these oligonucleotide primers are shown below. The underlined portions of the primers overlap with the I-domain sequence (see, ID SEQ NO: I). The other portions in KAC249 and KAC250 provide the N-terminus extension sequence for the I-domain and the restriction enzyme BamHI site for cloning of the I-domain into the vector pGEX-3X or pGEX-2T. The unlined portion in KAC251 provides a protein synthesis stop codon TAG to terminate the I-domain sequence at glu337 and the restriction enzyme EcoRI site for cloning into the vectors.

KAC249: 5' GGCTCGGATCCGGTGGCAGTGACATTGCCTTCTTGATTGATGGCTCT

KAC250: 5'

GGCTCGGGATCCCCGGTGGCAGTGACATTGCCTTCTTGATTGATGGC

KAC251: 5' GAGCCTGAATTCTATTCCTGAGACATCTCATGCTCAAAGGAGCT

The PCR reaction mixture, in a final volume of 100 µl, was composed of 68 µl water, 10 µl 10x reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 16 µl of a mixture of the four deoxynucleotide triphosphates (each at 1.25 mM), 1 µM each of the appropriate oligonucleotide primer, 1 ng pET-CD11b/I$(His)_6$ plasmid DNA, and 1 µl of Taq polymerase. The reaction was carried out by application of the GeneAmp PCR system (Cetus) in the following manner: The sample was heated at 94^0C , then treated for 30 cycles of 94^0C for 30 seconds, 65^0C for 30 seconds, and 72^0C for 45 seconds, followed by a 5 minute anneal at 65^0C and a 5 minute extension at 72^0C, and then cooled to 4^0C.

An aliquot of the PCR reaction mixture was analyzed by 1.5% agarose gel electrophoresis to confirm that the size of the DNA fragment is about 660 bp. The DNA fragment was then purified by phenol extraction and precipitated from the PCR reaction mixture by the addition of 100 µl of 0.3 M sodium acetate and 400 µl ethanol. This 660 bp DNA fragment was treated with BamHI and EcoRI, purified by 1.5% agarose gel electrophoresis, purified by phenol extraction and ethanol precipitation, and stored at -20^0C in 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA until use for cloning into expression vectors.

Expression of the CD11b I-domain Fusion to GST

Expression vectors pGST-X^a-CD11b/I and pGST-Throm-CD11b/I were transformed into 3 different *E. coli* hosts (JM103 and JM109 are commercially available; K12S was isolated to test for expression of the fusion protein). Induction of the tac promoter to express the fusion protein was by the addition of IPTG (isopropylthiogalactoside) at $1x10^{-3}$ M or as specified. The *E. coli* cells were grown at 37^0C with aeration in growth medium (Luria Broth with 100 µg/ml ampicillin). Specifically, an overnight culture of *E. coli* cells was diluted 50-100 fold with growth medium to A550 of about 0.1, allowed to grow until mid-log phase (A550 0.6-0.8), and then induced by the addition of IPTG. At the end of induction (2-4 hours) cells were collected by centrifugation, resupended to A550 of 20 with 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, and stored at -20^0C until further use. Sonicated cell extracts were prepared by sonication of the cell suspension in a Branson Sonifier.

To determine the level of expression, sonicated cell extracts were analyzed by SDS-PAGE and the gel with the Coomassie stained protein bands was scanned by a Shimadzu densitometer. The amount of the fusion protein in the cell extract was calculated as a percent of total cell protein. SDS-PAGE was carried out using a gel system where the cross linker is N,N'-diallytartar diamide (Morse *et al*, Anatomy of herpes simplex virus DNA, J. Virol., **26**:389-410 (1978) at a polyacrylamide

concentration of 17%. Protein molecular weight standards were purchased from Amersham.

Preparation of Inclusion Body-Free Extracts

The soluble fusion protein found in the cytoplasm of the *E. coli* cells was used as the starting material for downstream purification of the I-domain. To remove the insoluble, aggregated form of the fusion protein (in the inclusion bodies), sonicated cell extracts were centrifuged in the Sorvall RC-5B centrifuge with the SS34 rotor at 13,000xg for 5 minutes at 4^0C. The inclusion body-free supernatant was stored at -20^0C until further use.

Preparation of Medium for ^{15}N and ^{15}N/^{13}C Labeling of I-domain

E. coli cells to produce ^{15}N- and ^{15}N^{13}C-labeled I-domain, were cultured in medium purchased form Martek Bioscience Corp., Columbia, Maryland. The medium was diluted with M9 salts medium, Sambrook *et al.* (cited, above). To test the approriate dilution ratio, the Martek non-labeled medium Celtone-U was used with M9 salts medium without supplement of amino acids. Ampicillin at 100 µg/ml was used and cell growth was at 37^0C. To label the proteins with ^{15}N, cells were grown in Martek Celtone-N medium (>98% ^{15}N) diluted with 2 parts of M9 salts medium in which 2 gm/l of ^{15}N-ammonium chloride was used as the sole ammonium source. To label the proteins with ^{15}N and ^{13}C, cells were grown in Martek Celtone-CN medium (>98% ^{13}C and ^{15}N) diluted with 2 parts of M9 salts medium in which 2 gm/l of ^{15}N-ammonium chloride and 4 gm/l of ^{13}C-glucose were used as the sole ammonium and carbon source.

Methods: Expression of GST-I-domain

The subject I-domain, generated from this fusion protein by cleavage with Factor X^a, corresponds exactly to residues 133 through 337 of *mature* Mac-1, plus a pentapeptide N-terminal extension, Gly-Ile-Pro-Gly-Gly, required to enable the proteolytic cleavage. A schematic comparison of the I-domain structure with that published by Michishita *et al.*(cited, above) for the A-domain, is given in Figure 1. The subject protein begins and ends about 20-residues downstream from the A-domain (Fig. 1). This is a significant difference, and can account for differences which might be seen in the function of these proteins. Moreover, the subject I-domain has an N-terminal extension, whereas the A-domain has a COOH-terminal segment, neither of which belong to the protein in question.

Purification of I-domain: Glutathione Sepharose Chromatography

Glutathione-Sepharose and S-Sepharose Fast Flow were purchased from Pharmacia LKB. Reduced glutathione (GSH) was purchased from Sigma.

Sequencing-grade Factor X^a was obtained from Boehringer Mannheim. Ultrafiltration membranes were from Amicon and polyacrylamide gels were purchased from ISS Enprotech. All other reagents were of the highest quality commercially available. *E. coli* cell homogenates were centrifuged at 40,000 x g and subsequently filtered through a 0.45 μm filter. The filtrate was loaded directly onto a Glutathione Sepharose column (50-ml; 1.6cm i.d. x 26cm length) pre-equilibrated in PBS/0.2% β-octyl glucoside, pH 7.4. The column was loaded at 1.0 ml/min and protein in the effluent was monitored at 280 nm. The column was washed with 4 column volumes (CV) of equilibration buffer and step-eluted with 2 CV of 50 mM Tris buffer, pH 8.0 containing 20 mM reduced glutathione. The eluent was collected in 9-ml fractions. All process fractions were subjected to SDS-PAGE under non-reducing conditions (NR). Fractions containing GST/I-domain were pooled and stored at 4 °C.

Factor X^a Cleavage

Purified fusion protein was subjected to digestion with Factor X^a (1% by weight) for 16-24 hours at 23°C. SDS-PAGE was always run to confirm that digestion was complete.

S-Sepharose Fast Flow Chromatography

The protein digest was loaded directly onto an S-Sepharose Fast Flow column (50-ml; 1.6cm i.d. x 26cm length) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.5. The column was run at 1.0 ml/min and the effluent monitored at 280 nm. The column was washed with 4 CV of equilibration buffer, and eluted with a 2 CV linear salt gradient run from 0-1.0 M NaCl in the presence of 50 mM sodium phosphate, pH 6.5. Column fractions were subjected to SDS-PAGE (NR). Purified I-domain was observed as a single band at approximately 25 kD, and eluted from the column at approximately 150 mM NaCl.

Ultrafiltration

Purified I-domain was concentrated to 20 mg/ml using an Amicon stirred-cell ultrafiltration module containing a YM05 membrane. Nitrogen pressure was maintained at 60 psi during the concentration.

Characterization of I-domain; Analytical Methods

Proteins were sequenced by automated Edman degradation in an Applied Biosystems Model 470 Gas Phase Sequencer fitted with an on-line HPLC analyzer (Model 120A) for identification and quantitation of phenylthiohydantoin (PTH) amino acids. Integration of the peaks from the HPLC was performed with a Nelson Analytical 3000 Series chromatography data system connected in parallel with the

recorder to the output of the Model 120A HPLC system.

Amino acid analysis was performed with the aid of a Beckman Model 6300 analyzer. Samples were hydrolyzed for 24 hours in vacuo in 6N HCl at 110 °C, followed by drying in a Speed Vac Concentrator (Savant). Resulting residues were reconstituted in buffer (NaS; Beckman) and applied to the analyzer.

Reversed-Phase (RP) HPLC was performed on an HP 1090 liquid chromatograph with a 4.6 x 250 mm Vydac C4 column. Gradient mobile phases were water and acetonitrile, each containing 0.15% trifluoroacetic acid (TFA). The gradient was run from 0-70% acetonitrile in 70 minutes with a flow rate of 1.0 ml/min. The effluent was monitored simultaneously at 220 nm and 280 nm. The I-domain eluted as a single peak, and was collected in 2-ml polypropylene microfuge tubes, dried in a Speed Vac, and analyzed. SDS-PAGE was run as described by Laemmli, Nature, 277:680-85 (1970), or using the tricine buffer system as described by Schagger and von Jagow, Anal. BioChem., 166:368-79 (1987). Gels were fixed and subsequently stained with 0.1% Coomassie Brilliant Blue R-250.

All electrospray ionization (ESI) mass spectra were recorded on the Vestec 201A mass spectrometer. Aliquots (5 µl) of a solution of the I-domain in acetonitrile/water/0.1% TFA (about 0.05 to 0.5 µg of protein isolated by HPLC in the acetonitrile/TFA gradient system) were injected via a loop injector into the ion source. The mass spectrometer was scanned from m/z 500 to 2000 at 2 sec/scan. The data were acquired with the Teknivent Vector 2 data system. Ten scans were averaged and transfered to the Harris 800 computer for further processing. The average molecular weights (av. M.W.) were determined using programs developed in-house. For single components, the centroid program and the deconvolution program were used. For mixtures, the deconvolution program yielded better results. The experimentally obtained average M.W. were then compared with the theoretical av. M.W. of the various samples of I-domain.

The circular dichroism (CD) spectrum of I-domain (1.0 mg/ml) was measured at room temperature (20-22 °C) on a Jasco Model J-720 CD spectropolarimeter from 260-190 nm in a 0.086 mm cell. The spectropolarimeter was calibrated at 290 nm with D-10-camphorsulfonic acid. Molar intensities were computed from the concentration of the protein sample and a mean residue molecular weight of 113.2. The secondary structure was calculated using the method of Compton and Johnson against a data base of 16 proteins.

Purification of I-domain

The *E. coli* expression system with plasmid pG-3x-CD11b provided reasonable

levels of GST/I-domain fusion protein. Purification of the GST/I-domain construct over immobilized GSH proved to be very straightforward, as good yields were observed with extremely high purities. In fact, the only contaminant observed upon SDS-PAGE analysis appeared to be a low level (<5%) of free GST, which would be expected to co-purify with the fusion protein. RP-HPLC of the protein recovered from the GSH-column yielded a single peak which, upon N-terminal sequence analysis yielded a single sequence corresponding to the N-terminus of GST. The absence of secondary sequences was good evidence that there was little or no proteolytic degradation of the fusion protein occurring during the first process step. Amino acid analysis of the RP-HPLC peak shows excellent correlation with the expected composition of intact GST/I-domain fusion protein. Based on these analytical results, the purity of the intact fusion protein was estimated to be >95%.

Liberation of the I-domain from the fusion protein was achieved enzymatically via incubation with Factor X^a. Factor X^a is a serine protease which, by design of the construct, should specifically cleave the fusion protein at the Arg-Gly bond leading into the Gly-Ile-Pro-Gly-Gly- N-terminal extension of the I-domain (SEQ. ID. NO. I , Fig. 1). Factor X^a was added directly to the purified fusion protein to a final concentration of 1% (by weight), and incubation was carried out at room temperature. An SDS-PAGE profile of the enzymatic timecourse of digestion with Factor X^a shows that digestion of the fusion protein is complete between 16-24 hours. Since GST and I-domain are nearly identical in molecular weight, these protein products are not resolved by this method. Nevertheless, SDS-PAGE provided a useful technique for monitoring the extent of digestion, since the loss of fusion protein could be followed easily.

Because the isoelectric points of GST (pI=6.5) and Factor X^a (pI=4.3) are significantly different from one another, and from that of the intact I-domain (pI=9.0), ion exchange chromatography was the final step in the purification of I-domain. The Factor X^a digest was loaded directly onto the cation exchange resin S-Sepharose Fast Flow under conditions that would allow only I-domain to bind. The I-domain eluted as a single homogeneous peak at around 150 mM NaCl in the gradient, resulting in its recovery in physiologic-type buffer. Efficiency of binding was monitored by RP-HPLC, which resolves GST from I-domain. The S-Sepharose load showed two peaks upon RP-HPLC (corresponding to GST and I-domain), while the flowthrough, wash, and pooled fractions each contained predominantly one peak corresponding to GST, GST, and I-domain, respectively.

Characterization of I-domain

N-terminal sequence analysis and amino acid analysis performed on the final product resulted in very high correlations to expected results with no hint of contamination or degradation. Upon overloading a sample of this pool for SDS-PAGE, two minor bands are observed (≈9kD and ≈15kD). RP-HPLC analysis also shows the presence of two early-eluting peaks at low levels (2-3%). Collection, lyophilization, and SDS-PAGE analysis of these peaks confirms that they are the ≈9kD and ≈15kD species. N-terminal sequencing and amino acid analysis of both peaks shows that they are degradation products of the I-domain resulting from a single internal cleavage at Arg residue 306 (SEQ ID NO: I). Based on these results, the purity of I-domain derived from this process has been determined to be ≥95%. Material of this high quality has been obtained consistently by means of this purification protocol (n=7).

Electrospray ionization mass spectrometry was used to further assess the integrity of the I-domain preparations. Purified I-domain (2-3 nmoles) was collected from RP-HPLC, lyophilized, and reconstituted in a small volume of 50% acetonitrile prior to application to the spectrometer probe. A series of molecular ions corresponding to mass per unit charge (m/z) was obtained, from which the average molecular weight can be determined. All Factor X^a-derived I-domain preparations yielded essentially identical results. The spectra identified two species with distinct molecular weights. The molecular weight of the major species (80-95%) was determined to be 23,767, which matches the theoretical value exactly, while the minor species yielded a mass 164 daltons lower. Generation of this species does not appear to be due to C-terminal heterogeneity or typical post-translational modification based on this mass difference.

The monoclonal antibody (mAb) 3H5 directed against CD11b was generated, isolated and characterized. Fibrinogen was purchased from Kabi Pharmacia (Franklin, Ohio) and was fibronectin depleted utilizing gelatin-Sepharose 4B (Pharmacia; Uppsala, Sweden).

Cell Preparation

Polymorphonuclear leukocytes (PMNs) were isolated from heparinized blood by dextran-sedimentation and Ficoll-Hypaque gradient centrifugation as previously described (Smith, C.W., et al., J. Clin. Invest., 83:2008-17 (1989)). PMNs were washed 2 times in phosphate-buffered saline (1.2 mM phosphate, 138 mM NaCl, pH 7.4; PBS) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. PMNS were labeled with 2′, 7′-*bis*-(2--carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF; Molecular Probes,

Inc., Eugene, OR; 1 mg/ml in 90% DMSO).

Cell Adhesion Assays:

Neutrophil Adhesion to Protein Substrates in Microtiter Plates (Tables 1, 2, & 3)

To evaluate neutrophil adhesion to iC3b ligands, 96 well microtiter plates (Immulon 2, Dynatech Labs, Chantilly, VA) were coated with 200 µl of PBS containing 170 µg/ml BSA and 75 µg/ml human polyclonal IgG (Calbiochem, La Jolla, CA). Following incubation for 1 hour at 37°C, plates were washed three times with 200 µl PBS. The remaining sites on the plastic were blocked by addition of 1% gelatin in PBS. Plates were washed once with 200 µl of PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/Ca^{2+}/Mg^{2+}) followed by the addition of 200 µl of human serum (diluted 1:3 in PBS/Ca^{2+}/Mg^{2+}. Plates were washed three times with 200 µl of PBS containing 0.05% Tween 20 and 0.01% thimerosal.

Labeled neutrophils were stimulated with n-formyl-methione-leucine-phenylalanine (fMLP; 10^{-7} M) for 10 min at 37°C. Fifty µl of the anti CD11b mAb 3H5 (positive control) (20 µg/ml) or I-domain protein (0.5, 1, 5, 10, or 20 µM) was added to some of the wells before addition of 50 µl of neutrophils (10^7/ml). Following 20 minutes incubation at 37°C, nonadherent cells were removed by flicking the plates. Plates were then washed three times with 200 µl PBS/Ca^{2+}/Mg^{2+}. The amount of fluorescence in the wells was measured at 485/535 nm using a Pandex fluorescence concentration analyzer (Baxter Healthcare Corp., Mundelein, IL).

To evaluate neutrophil adhesion to fibrinogen, 96 well microtiter plates were coated with 50 µl of fibrinogen (100 µg/ml) for 2 hours at 37°C. The remaining sites on the plastic were blocked by addition of 1% gelatin in PBS. Plates were washed three times with PBS/Ca^{2+}/Mg^{2+}. Fifty µl of mAb 3H5 (20 µg/ml) or I-domain protein (0.5, 1, 5, 10, or 20 µM) was added to some of the wells before addition of cells. fMLP stimulated labeled neutrophils were added to the wells and incubated for 20 minutes at 37°C, and cell adherence was determined as described above.

In order to evaluate neutrophil adherence to ICAM-1, this protein was immunopurified from 70 grams of human placenta using a modification of a procedure as described (Marlin and Springer, Purified intercellular adhesion molecule-1(ICAM-1), Cell., **51**:813-19 (1987)). The anti ICAM-1 mAb 8.4 (*) was used for these affinity chromatography procedures. For neutrophil adhesion assays, microtiter plates were coated with purified ICAM-1 diluted 1:15 (vol./vol.) in PBS/Ca^{2+}/Mg^{2+}. After a 2 hour incubation at 37°C, ICAM-1 substrates were blocked by addition of 1% gelatin in PBS. Plates were washed x 3 with

PBS/Ca^{2+}/Mg^{2+}. Fifty µl of mAb 3H5 (20 µl/ml) of I-domain protein (0.5, 1, 5, 10 or 20 µM) was added to some wells before addition of cells. fMLP stimulated labeled neutrophils were added to wells and incubated for 20 minutes at 37°C and cell adherence was determined as described above.

Neutrophil adhesion to protein substrates in Smith-Hollers adhesion chambers (Table 4)

In addition to the microtiter plate assays (above), adhesion of chemotactically stimulated neutrophils to Mac-1 substrates was assayed in Smith-Hollers chambers as previously described (Anderson et al., Abnormal mobility of neonatal polymorphonucler leukocytes, J. Clin. Invest. **68**:863-74 (1981)). Use of these adhesion chambers facilitates analysis of adhesion in the absence of shear stress, conditions which enhance β_2 integrin-dependence (Lawrence *et al.*, Effect of venous shear stress on CD-18..., Blood, **75**:227-37 (1989)). Neutrophils (10^7/ml) were preincubated in suspensions containing fMLP (10 nM) and I-domain (2.5 - 25 µM) or in the CD11b mAb 3H5 (10 µg/ml) for 5 minutes at 21°C prior to incorporation in adhesion chambers (0.5 x 10^6). For preparation of protein substrates, KLH (1 mg/ml) or fibrinogen (10 mg/ml) were incubated on glass coverslips for 30 min at 37°C and washed prior to assay. The iC3b substrates were prepared by incubating a human polyclonal IgG (75 µg/ml) - BSA (170 µg/ml) mixture for 1 hour at 37°C on glass coverslips prior to adding human serum (dilutes 1:3 in PBS) and incubating for an additional 1 hour at 37°C. The percentages of adherent neutrophils contacting protein substrates were calculated as described (Anderson *et al,*(1981), cited above).

Neutrophil-endothelial adhesion and migration assays (Table 5, 6)

Assessments of neutrophil adhesion to and migration through confluent monolayers of human umbilical vein endothelial cells (HUVEC) were performed in Smith-Hollers adhesion chambers as described (Smith *et al.*, Cooperative Interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1..., J. Clin. Invest. **83**:2008-17 (1989)). For the representative experiments illustrated in Tables 4 and 5, HUVEC were preincubated with TNF (100 U/ml, 4 h, 37°C) to induce high levels of ICAM-1 expression prior to incorporation into chambers. Neutrophils (10^7/ml) were preincubated with selected concentrations of CD11b I-domain (Factor X_a-cleaved GST fusion protein), GST, or the anti CD11b mAb 3H5 (10 µg/ml) for 5 min at 37°C prior to incorporation in adhesion assays (0.7 ml/chamber). The percentage of adherent neutrophils contacting HUVEC and the percentage of cells xhibiting transendothelial migration were determined as previously described (Smith, C.W. *et al.*, J. Clin. Invest. **82**:1746-1756, (1988).

ICAM-1 Purification

ICAM-1 was purified from 70 grams of human placenta by a modified procedure described (Marlin, S., Springer, T.A., Cell, **51**:813-19 (1987)). The anti-ICAM-1 monoclonal antibody 8.4 was used for the affinity chromatography purification.

TABLE 1

Adhesion of Neutrophils to iC3b

I-Domain Conc. (μM)	6-His I-domain	Factor X	6-His peptide
20	627	35484	38500
10	730	37561	41216
5	762	32640	35450
1	15027	28402	36387
.5	24456	32177	40912
0	27322	N.D.	36876
	3H5 monoclonal Ab. 8570		

Table 1. Adhesion of neutrophils to iC3b.

Fluorescently labeled neutrophils and I-domain constructs (at the indicated concentrations) were added to microtiter wells that had been coated with iC3b as described under "Materials and Methods". Following a 20 minute incubation, nonadherent neutrophils were removed by flicking and the microtiter wells were washed three times with assay buffer. Adherent cells were quantitated by measuring the fluorescent intensity using a Pandex fluorescence concentration analyzer. The adhesion of neutrophils was also determined in the presence of mAb 3H5 (anti-CD11b). Each value (expressed as fluorescence intensity) represents the mean ± standard error of three determinations. Results are representative of three experiments. The 6-His I-Domain is an extension at the COOH-terminus of six histidines (6His tag) to facilitate purification by immobilized metal ion affinity chromatography. This construct begins with H_2N-Ser_{133}-Asp-Ile-Ala-Phe-Leu..., and ends with ..Ser-Gln-Glu_{337}-Ile-Leu-Gly-Arg-↓-His-His-His-His-His-His-COOH, where the numbers indicate residues in the Mac-1 α-subunit (from, Michishita *et al*, Cell, 72:857-67 (1993), correspondes to Ser 231-Glu 435, SEQ ID NO: 1), and the arrow indicates a bond designed, by virture of the preceeding 4 amino acids, to be a cleavage point for Factor X^a. The 6-His peptide is a control for the 6-His I-Domain starting Ser-Gln-Glu-Ile-Leu-Gly-Arg-↓-His-His-His-His-His-His-COOH to assure that the observed activity of the 6-His I-Domain is not an artifact.

These results indicate that the 6-His-I-Domain protein inhibits neutrophil adhesion to iC3b in a dose-dependent manner. Half maximal inhibition was observed at a concentration of 1 micromolor. This inhibition does not appear to be due to the 6-His tag itself, since the control peptide did not inhibit adhesion. In contrast, the Factor X (never frozen) cleaved I-Domain protein did not inhibit neutrophil adhesion to iC3b even at the highest concentration tested (20 micromolar). Attachment was specific, since it was inhibited by mAb 3H5 (anti-CD11b).

TABLE 2

Adhesion of Neutrophils to Fibrinogen

I-Domain conc. (μM)	6-His I-Domain	Factor X*	Factor X**	6-His peptide
20	34917	52056	16790	44616
10	30949	43282	15110	47685
5	38270	54150	19461	51523
1	37002	42670	26798	46818
.5	47019	46423	48530	47230
0	40854	41588	49273	38952
	3H5 monoclonal Ab. 18906			

* previously frozen material

**never frozen

Table 2. Adhesion of neutrophils to fibrinogen.

Fluorescently labeled neutrophils and I-domain constructs (at the indicated concentrations) were added to microtiter wells that had been coated with fibrinogen (100 μl/ml). Following a 20 minute incubation, nonadherent neutrophils were removed by flicking and the microtiter wells were washed three times with assay buffer. Adherent cells were quantitated by measuring the fluorescent intensity using a Pandex fluorescence concentration analyzer. The adhesion of neutrophils was also determined in the presence of mAb 3H5 (anti-CD11b). Each value (fluorescence intensity) represents the mean ± standard error of three determinations. Results are representative of three experiments.

The results indicate that the Factor X cleaved GST-I-Domain fusion protein inhibited neutrophil adhesion in a dose-dependent manner such that half maximal inhibition was observed at a concentration of 1 micromolar. In contrast, neutrophil adhesion to fibrinogen was only marginally inhibited by the 6-His I-Domain fusion protein. The control peptide had no effect on adhesion and the previously frozen and thawed Factor X was inactive. Attachment was specific, since it was inhibited by mAb 3H5 (anti-CD11b).

TABLE 3

Adhesion of Neutrophils to ICAM-1

I-domain conc. (μM)	6-His I-domain	Factor X*	Factor X**	6-His peptide
20	8480	22156	11274	23274
10	9758	19187	9846	18008
5	13772	22040	12370	20502
1	18476	20895	14292	19977
.5	19401	19081	15599	16546
0	19977	20587	18258	14894
	3H5 monoclonal Ab. 10465			

*previously frozen material; ** never frozen

Table 3. Neutrophil adhesion to ICAM-1.

Fluorescently labeled neutrophils and I-domain constructs (at the indicated concentrations) were added to microtiter wells that had been coated with purified placental ICAM-1. Following a 20-minute incubation, nonadherent neutrophils were removed by flicking and the microtiter wells were washed three times with assay buffer. Adherent cells were quantitated by measuring the fluorescent intensity using a Pandex fluorescence concentration analyzer. The adhesion of neutrophils was also determined in the presence of mAb 3H5 (anti-CD11b). Each value (fluorescence intensity) represents the mean ± standard error of three determinations.

The results demonstrate that both the Factor X and the 6-His tag I-Domain fusion proteins inhibited neutrophil adhesion to purified ICAM-1 in a dose-dependent manner. Half maximal inhibition was observed at concentrations of 1 micromolar and 3 micromolar, respectively. The control peptide had no effect on adhesion and the frozen and thawed Factor X was inactive. Attachment was specific since it was inhibited by mAb 3H5 (anti-CD11b).

In summary, Tables 1-3 show that CD11b I-Domain proteins have differential effects on neutrophil adhesion. The Factor X I-Domain inhibits neutrophil adhesion

to fibrinogen and ICAM-1 in a dose-dependent manner, but has no inhibitory effect on neutrophil adhesion to iC3b. Half maximal inhibition to fibrinogen and ICAM-1 is observed at a concentration of 1 micromolar for both proteins. In contrast, the 6-His tag I-domain protein blocks neutrophil adhesion to ligands iC3b and ICAM-1 in a dose-dependent manner such that half maximal inhibition occurs at concentrations of 1 and 3 micromolar, respectively. A control peptide containing hexahistidine had no effect on neutrophil adhesion. These results further support the importance of the I-domain in ligand binding function of CD11b/CD18.

TABLE 4
Effect of CD11b I-Domain (Factor X_a cleaved GST fusion protein) on neutrophil adhesion to protein substrates

Substrate+	Neutrophil-Pretreatment*	Adhesion (%)** (n = 2)	% Inhibition by I-domain or mAb
KLH	fMLP 10 nM, PBS	81	--
	" , I-Domain (25 µM)	60	26
	" , I-Domain (10 µM)	71	14
	" , I-Domain (2.5 µM)	61	25
	" , 3H5 mAb (10 µg/ml)	23	71
iC3b	" , PBS	97	--
	" , I-Domain (25 µM)	98	--
	" , I-Domain (2.5 µM)	60	39
	" , 3H5 mAb (10 µg/ml)	47	51
Fibrinogen	" , PBS	72	--
	" , I-Domain (25 µM)	49	32
	" , I-Domain (10 µM)	44	39
	" , I-Domain (2.5 µM)	34	53
	" , 3H5 mAb (10 µg/ml)	21	71

+ KLH (1 mg/ml) or fibrinogen (10 µg/ml) were incubated on glass coverslips for 30 min. at 37°C and washed prior to assay. The iC3b substrates were prepared by incubating an IgG (75 µg/ml) - BSA (170 µg/ml) mixture for 1 hr. at 37°C on glass coverslips prior to adding a 1:3 dilution of human serum and incubating an additional 1 hr. at 37°C.

* Neutrophils were preincubated in fMLP and I-domain or mAb for 5 min. at 21°C prior to incorporation into Smith-Hollers adhesion chambers. Final concentrations of proteins are designated.

** Neutrophil adhesion was assayed as described (Anderson et al., Abnormal mobility of neonatal polymorphonucler leukocytes, J. Clin. Invest. 68:863-74 (1981)).

Results of the representative experiment (above) indicate the capacity of the CD11b I-Domain protein to inhibit stimulated neutrophil adhesion to KLH, iC3b and/or fibrinogen substrates when assessed in the absence of shear stress. This is especially apparent with respect to iC3b and fibrinogen binding, where 2.5 µM and/or 10 µM concentrations of I-domain inhibit adhesion by 39-53% as compared to stimulated controls. This level of inhibition is comparable to that of the blocking mAb 3H5 directed at Mac-1 (CD11b) when used at saturating concentrations (10 µg/ml). An apparent concentration-independent relationship for I-Domain inhibition is observed. The optimum inhibitory concentration of I-Domain is 2.5 µM.

TABLE 5
Effect of CD11b I-Domain (Factor X_a-cleaved GST fusion protein) on neutrophil-endothelial adhesion

Pretreatment Conditions		Total Adhesion (%)**	% Inhibition
HUVEC	Neutrophils*		
--	--	11 ± 2	
TNF 100 U/ml, 4 hr, 37°	--	88 ± 6	--
"	I-Domain (25 µM)	68 ± 4	24
"	I-Domain (5 µM)	56 ± 4	37
"	3H5 anti CD11b mAb (10 µg/ml)	39 ± 3	56

* Neutrophils were preincubated with I-domain or mAb for 5 min. at 21°C prior to incorporation into Smith-Hollers adhesion chambers. Final concentrations of proteins are desginated.

** Assay performed as described by Smith, C.W. *et al.*, J. Clin. Invest. **82**:1745-1756, (1988).

Results of the representative experiment shown above demonstrate the capacity of CD11b - I-Domain to inhibit the adhesion of unstimulated neutrophils to endothelial monolayers pretreated with TNF to maximallly induce expression of ICAM-1 and other neutrophil adhesion ligands. As shown, final I-Domain concentrations of 5 µM or 25 µM inhibited adhesion (as compared to stimulated controls) by 37% or 24%, respectively, while the positive control 3H5 mAb inhibited adhesion by 56% when used at saturating concentrations. The remaining adhesion (unblocked by 3H5 mAb) represents the contribution of E-selectin and other endothelial ligands elicited by TNF and is not expected to be impacted by I-Domain protein in this assay.

TABLE 6
Effect of CD11b I-Domain (Factor X_a-cleaved GST fusion protein) on neutrophil-endothelial adhesion: Experiment #2

Pretreatment Conditions		Total Adhesion (%)**	Transendothelial Migration (%)**
HUVEC	Neutrophils		
--	--	1 ± 2	0
TNF 100 U/ml, 4 hrs, 37°C	--	79 ± 7	20 ± 4
"	I-Domain (25 µM)	75 ± 6	5 ± 2
"	I-Domain (10 µM)	62 ± 5	15 ± 3
"	I-Domain (5 µM)	49 ± 4	16 ± 4
"	I-Domain (1 µM)	18 ± 3	5 ± 2
"	3H5 (anti CD11b) mAb (10 µg/ml)	21 ± 3	5 ± 1
"	GST (25 µM)	82 ± 6	24 ± 6
"	GST (5 µM)	88 ± 6	20 ± 4

*. Neutrophils preincubated with I-domain, mAb or GST protein for 5 min at 21°C prior to incorporation in Smith-Hollers adhesion chambers. Final concentrations of proteins are designated.

** Neutrophil adhesion and transendothelial migration assayed as described by Smith, C.W. *et al.*, J. Clin. Invest. **82**:1746-1756, (1988).

Results of the representative experiment shown above illustrate the capacity of CD11b - I-Domain to inhibit both neutrophil adhesion to and migration through endothelial monolayers prestimulated with TNF. An inverse relationship between I-domain concentration and degree of inhibition of adhesion is seen. The optimal inhibitory concentration is 1 µM and in this experiment a 25 µM concentration is non inhibitory for adhesion. Importantly, a 1 µM concentration of I-Domain inhibits adhesion to the same extent as seen with the 3H5 mAb when used at saturating concentrations. The GST (control) fusion protein fragment is non inhibitory. Somewhat different relationships were observed in migration assessments. A bimodal dose response effect is apparent in which high concentrations (25 µM) of I-Domain block neutrophil motility independent of adhesion, and low concentrations (1 µM) block migration associated with diminished neutrophil-endothelial adhesion. In each case, transendothelial migration is potently inhibited to the same extent as effected by the 3H5 mAb. These findings indicate distinct inhibitory influences of CD11b - I-Domain protein on neutrophil motility (chemotaxis) and on adhesion *per se*. Such observations are entirely consistent with the known multifunctional properties of Mac-1 (Anderson *et al.*, Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions..., J. Immunol. 137:15-27 (1986) and Smith *et al.*, Transendothelial migration, In Adhesion. pp 85-115, W.E.Freeman and Co. NY (1992).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Upjohn Company
Heinrikson, Robert L.
Anderson, Donald C.
Tomich, Che-Shen C.
Fairbanks, Michael B.
Bajt, Mary L.

(ii) TITLE OF INVENTION: MAC-1 I-DOMAIN PROTEIN USEFUL IN BLOCKING ADHESION AND MIGRATION OF NEUTROPHILS

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 49001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Gateway 2000 P5-90
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Darnley, James D., Jr.
(B) REGISTRATION NUMBER: 33,673
(C) REFERENCE/DOCKET NUMBER: 4767.P CN1

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 616/385-6897
(C) TELEX: 224401

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 435 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro
1 5 10 15

Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
20 25 30

```
Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
        35                  40                  45

Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
    50                  55                  60

Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65                  70                  75                  80

Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
                85                  90                  95

Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
            100                 105                 110

Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
        115                 120                 125

Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
    130                 135                 140

Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145                 150                 155                 160

Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
                165                 170                 175

Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
            180                 185                 190

Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
        195                 200                 205

Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Ile Glu Gly
    210                 215                 220

Arg Gly Ile Pro Gly Gly Ser Asp Ile Ala Phe Leu Ile Asp Gly Ser
225                 230                 235                 240

Gly Ser Ile Ile Pro His Asp Phe Arg Arg Met Lys Glu Phe Val Ser
                245                 250                 255

Thr Val Met Glu Gln Leu Lys Lys Ser Lys Thr Leu Phe Ser Leu Met
            260                 265                 270

Gln Tyr Ser Glu Glu Phe Arg Ile His Phe Thr Phe Lys Glu Phe Gln
        275                 280                 285

Asn Asn Pro Asn Pro Arg Ser Leu Val Lys Pro Ile Thr Gln Leu Leu
    290                 295                 300

Gly Arg Thr His Thr Ala Thr Gly Ile Arg Lys Val Val Arg Glu Leu
305                 310                 315                 320

Phe Asn Ile Thr Asn Gly Ala Arg Lys Asn Ala Phe Lys Ile Leu Val
                325                 330                 335

Val Ile Thr Asp Gly Glu Lys Phe Gly Asp Pro Leu Gly Tyr Glu Asp
            340                 345                 350

Val Ile Pro Glu Ala Asp Arg Glu Gly Val Ile Arg Tyr Val Ile Gly
        355                 360                 365

Val Gly Asp Ala Phe Arg Ser Glu Lys Ser Arg Gln Glu Leu Asn Thr
    370                 375                 380
```

Ile Ala Ser Lys Pro Pro Arg Asp His Val Phe Gln Val Asn Asn Phe
385 390 395 400

Glu Ala Leu Lys Thr Ile Gln Asn Gln Leu Arg Glu Lys Ile Phe Ala
405 410 415

Ile Glu Gly Thr Gln Thr Gly Ser Ser Ser Ser Phe Glu His Glu Met
420 425 430

Ser Gln Glu
435

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Leu Val Pro Arg Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Val Pro Arg Gly Ser Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ile Glu Gly Arg Gly Ile Pro Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Ile Pro Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCTCGGATC CGGTGGCAGT GACATTGCCT TCTTGATTGA TGGCTCT 47

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGCTCGGGAT CCCCGGTGGC AGTGACATTG CCTTCTTGAT TGATGGC 47

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGCCTGAAT TCTATTCCTG AGACATCTCA TGCTCAAAGG AGCT 44

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ile Leu Gly Arg His His His His His His
1 5 10

WHAT IS CLAIMED:

1. A fusion protein of glutathione-S-transferase (GST) and I-Domain derived from human leukocyte B_2-integrin Mac-1 in which said GST and said I-Domain are linked by a peptide segment containing a Factor X^a cleavage site as set forth in ID SEQ NO: I.

2. An I-Domain protein defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I.

3. A pharmaceutical composition comprising:

a) a recombinant I-Domain protein derived from human leukocyte B_2-integrin Mac-1 defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I; and

b) a pharmaceutically acceptable carrier or excipient.

4. The pharmaceutical composition of Claim 3 wherein said recombinant I-Domain is a fragment, analog or chemical derivative of said protein.

5. A method for treating inflammation comprising:
administering to a patient suffering from an inflammatory condition a pharmaceutically effective amount of an anti-inflammatory agent comprising a recombinant I-Domain protein derived from human leukocyte B_2-integrin Mac-1 defined by the amino acid sequence Gly 226 through Glu 435, inclusive, as set forth in ID SEQ NO: I.

6. The method of Claim 5 wherein said recombinant I-Domain protein is a fragment, analog or chemical derivative of said protein.

INTERNATIONAL SEARCH REPORT

Internat' Application No
PCT/US 95/04439

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N9/10 C07K14/705 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, vol. 72, no. 6, 26 March 1993 CELL PRESS,CAMBRIDGE,MA,US;, pages 857-867, M. MICHISHITA ET AL. 'A novel divalent cation-binding site in the A domain of the beta2 Integrin CR3 (CD11b/CD18) is essential for ligand binding' cited in the application the whole document --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1995	18.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENE, vol. 67, no. 1, 1988 ELSEVIER SCIENCE PUBLISHERS,B.V.,AMSTERDAM,NL;, pages 31-40, D.B. SMITH AND K.S. JOHNSON 'Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase' the whole document ---	1,2
Y	EP-A-0 364 690 (DANA FARBER CANCER INST INC) 25 April 1990 cited in the application the whole document ---	3-6
A	J. CELL BIOL., vol. 120, no. 2, January 1993 ROCKEFELLER UNIVERSITY PRESS,NY,US;, pages 545-556, M.S. DIAMOND AND T.A. SPRINGER 'A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophilic adhesion to ICAM-1 and fibrinogen' the whole document ---	1-6
A	J. CELL BIOL., vol. 120, no. 4, February 1993 ROCKEFELLER UNIVERSITY PRESS,NY,US;, pages 1031-1043, M.S. DIAMOND ET AL. 'The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands' cited in the application the whole document -----	1-6

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 95/04439

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0364690	25-04-90	AU-B-	638937	15-07-93
		AU-A-	4014489	01-03-90
		JP-A-	3047077	28-02-91
		PT-B-	91502	04-05-95
		AU-B-	638450	01-07-93
		AU-A-	5349990	09-10-90
		EP-A,B	0387701	19-09-90
		HU-A-	65843	28-07-94
		JP-T-	4504127	23-07-92
		NZ-A-	232868	26-05-95
		WO-A-	9010453	20-09-90

INTERNATIONAL SEARCH REPORT

Int[illegible]ional application No.
PCT/US 95/ 04439

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5,6
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 5,6 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.